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Structure Based Design of Protein Ligands: A Study of Antibody-like Scaffolds Targeted Against the Anthrax Toxin

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Abstract

We have adopted structure-based approaches to enhance the affinities of two single chain antibodies, scFv1 and scFv4, that bind to two different epitopes on the Protective Antigen (PA), a toxin from *Bacillus anthracis*. In one approach, we have modified scFv4 and re-engineered a novel antibody-like scaffold in which we have placed V_L on the N terminus and V_H on the C-terminus and joined them by a 10 amino-acid-long linker. This scaffold preserves the native V_L-V_H contact interface and the dispositions of the CDR loops. It binds to PA with 10 fold higher affinity than scFv4. In a second approach, we have created a bispecific ligand by covalently joining scFv1 and scFv4 by a flexible linker that supports simultaneous and synergistic binding of the two scFvs to PA. This bispecific scFv1-linker-scFv4 binds to PA with 10 fold higher affinity than the individual scFvs. The newly re-engineered antibody-like scaffold of scFv4 and scFv1-linker-scFv4 are expected to be potent inhibitors of PA binding to the host cells.

Introduction

Anthrax toxins are generally considered the most important factors contributing to the disease symptoms. The toxin system consists of three proteins: the protective antigen (PA), the edema factor (EF), and the lethal factor (LF) [1, 2]. The genes of these 83 KD proteins are encoded by the *anthrax* plasmid px01. A bipartite PA/EF or PA/LF system is required for the multi-step toxin pathogenesis. *First,* PA binds to the host cell. *Second,* PA is cleaved by furin protease into 20 and 63 KD fragments. *Third,* the 63 KD PA forms a heptameric pore through which EF or LF is translocated into the cytosol. *Finally,* the catalytic EF or LF enzyme causes cytotoxicity. EF, an adenylate cyclase, upregulates cAMP level whereas LF, a zinc protease, causes cell lysis [2-5].

Since PA binding to the cell defines the first step of toxin pathogenesis, design of protein ligands to specifically target the cell binding epitopes on PA appears to be logical step for preventing *anthrax* intoxication. Previously, we reported [6] the generation of two scFvs (1 and 4) by phage-display using a naïve phagemid library ($6X10^9$ diversity) [6].

scFv4 was found to be effective in blocking the cell binding of PA whereas scFv1 was rather ineffective. This implied that scFv1 and scFv4 bound to two different epitopes on PA. However, PA binding affinities of both the ligands were relatively low (equilibrium dissociation constant, $K_D \geq 100$nm). It is well recognized that protein ligands ought to have high affinities (i.e., $K_D \leq 10$nm) to be effective therapeutic agents against toxins. Here, we describe two structure-based approaches that can be utilized to enhance the binding affinities of scFvs against their target proteins. These approaches are illustrated using *anthrax* PA as the target protein.

Approaches

Single-chain antibodies (scFv) serve as convenient substitutes for mouse monoclonal antibodies (mAb)—see Figure 1a,b. In scFv (Figure 1b), the variable heavy (V_H) and light (V_L) are joined by a flexible glycine-rich linker, SSGGGGSSGGGSGGGGS.

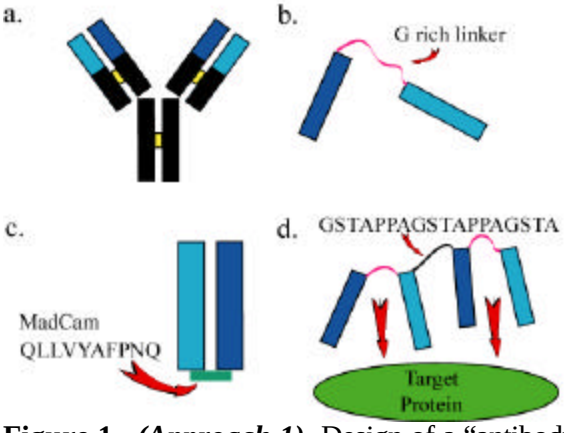

Figure 1. ***(Approach 1)*** Design of a "antibody-like scaffold" (c) that is a significant improvement over a conventional monoclonal antibody (a) or a single-chain antibody (b). ***(Approach 2)*** A bispecific ligand

(d) containing two single-chain antibodies that bind to two non-overlapping epitopes on a pathogen protein shows an enhanced binding affinity.

Due to presence of the same contact determining regions (CDRs), the scFv binds to the same antigen as the corresponding full-length mAb. However, the binding affinity of the scFv may not be the same as the full-length mAb due to (i) the relative disposition of V_H and V_L and (ii) the presence of the glycine-rich linker, both of which may disrupt the V_H-V_L contact interface as present in the full-length mAb-- compare Figure 1a and Figure 1b.

Approach 1: Design of an "antibody-like scaffold" We have carried out an extensive computer modeling to develop a strategy that allows us to retain (i) the native hydrophobic interactions between the V_L and V_H domains and (ii) the correct structure and exposure of the antigen binding (or CDR) loops. This involves covalent linkage of V_L on the N-terminus to V_H on the C-terminus by the MadCam linker, QLLVYAFPNQ—see Figure 1c. This sequence is present in the cell surface receptor as a natural linker between two immunoglobuline (V_L/V_H) like folds [7, 8]. As shown later, such an antibody-like scaffold is a significant improvement over single-chain antibodies.

Approach 2: Design of a bispecific protein ligand In this approach, we have relied upon identifying two protein ligands (such as scFvs) that bind to two non-overlapping epitopes on a given target protein. We have then joined these two ligands by a flexible linker, GSTAPPAGSTAPPAGSTA that supports simultaneous and synergistic binding of the two ligands to the target protein—see Figure 1d. The bispecific ligand shows significantly higher binding affinity than the individual ligands it is made of.

Results

Design of an "antibody-like scaffold" to improve the affinity of scFv4. V_L and V_H domains of scFv4 were joined by the MadCam linker, QLLVYAFPNQ. This also involved small truncation of V_L and V_H on the C- and N-termini respectively. Simulated annealing was performed on the newly constructed antibody-like scaffold using the SANDER module in AMBER (version 4.1). From the sampled energy landscape the low-energy scaffolds were screened subject to the following criteria: (i) the native hydrophobic interactions between the V_L and V_H domains and (ii) the correct structure and exposure of the antigen binding (or CDR) loops. Figure 2 shows a low-energy structure of such a scaffold which targets the cell binding epitope on PA.

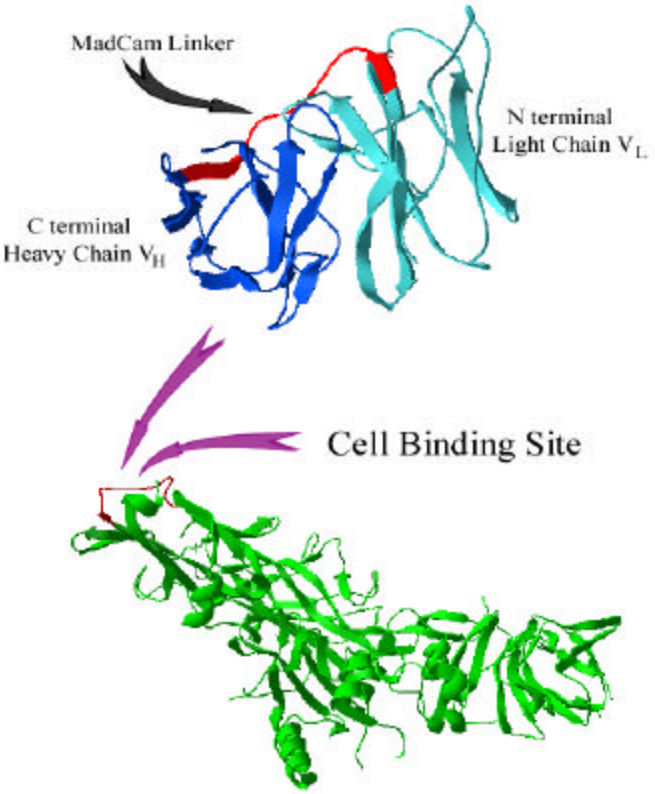

Figure 2. An antibody-like scaffold constructed from scFv4. This scaffold targets the cell-binding site on PA.

MadCam linked scFv4 or MCscFv4 (i.e., the construct in Figure 2) was cloned into pET27b vector and overexpressed using 2mM IPTG for 5 hours. The protein was eluted from the insoluble fraction with 8M urea and purified using a 6XHis tag and Talon (Clonetech) resin. 100mM imidazole was used to elute MCscFv4 from the resin. Purified MCscFv4 was dialyzed into PBS.

The purified MCscFv4 was tested for its binding to PA by flow cytometry. The equilibrium dissociation constant, K_D, of MCscFv4 for PA binding was ~15nM which is about 20-fold higher than the PA binding affinity of scFv4.

Design of a bispecific ligand using PA-specific scFv1 and scFv4. We have also developed a general cloning strategy to construct bispecific ligands using

two antibody-like genes such as scFvs or antibody-like scaffolds shown in Figure 1. Briefly, two synthetic scFv genes (A and B) are constructed with two unique restriction sites respectively on their C and N terminal coding sites. This allows joining of any linker to obtain a bispecific scFvA-linker-scFvB (see Figure 1d). We have been successful in covalently linking them to design, overexpress in *E. coli*, and purify a high affinity bispecific antibody-like scaffold against PA.

Molecular modeling suggested that GSTAPPAGSTAPPAGSTA is the optimum linker sequence between scFv1 and scFv4. The synthetic gene encoding scFv1-linker-scFv4 was cloned into pET27b vector and overexpressed using 2mM IPTG for 5 hours. The protein was eluted from the soluble fraction and purified using a 6XHis tag and Talon (Clonetech) resin. 100mM imidazole was used to elute the protein from the resin. Purified scFv1-linker-scFv4 was dialysed into PBS.

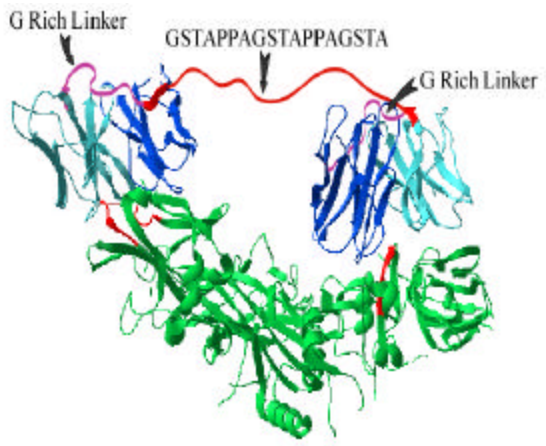

Figure 3. Construction of bispecific ligand using the PA-specific scFv1 and scFv4.

As shown in Figure 3, this ligand binds to two non-overlapping epitopes on PA. Flow cytometry data show that the purified bispecific antibody-like scaffold binds to PA with a K_D~10nM, whereas the individual scFvs bind to PA with 10-fold lesser affinities, i.e., $K_D \geq 100$nM.

The bispecific ligand was also tested by flow cytometry for its ability to block the binding of PA to murine and human macrophages. Indeed, it effectively blocked the binding of PA to macrophages.

Discussion

The strategy for generating antibody-like scaffolds can easily be extended to target any surface epitope on any pathogen protein. For this, we will compile a database of antibody-antigen pairs in which the CDR loops of the antibodies will be correlated with the antigen sequences to which they bind. We will use this database to develop an *in vitro* selection or a structure-based method to generate antibody-like scaffolds.

Since antigen-antibody specificity is chiefly governed by the 6 CDR loops (3 each in the heavy and light chains), we will identify different combinations of CDR loops that lead to different antigen specificities from the database. This will allow us to design and synthesize the genes of antibody-like scaffolds for a variety of known antigens. Starting from this limited but distinctly different scaffolds, we will generate a library of scaffolds by shuffling the CDR loops without changing the skeleton of the scaffold. We will then develop an ELISA based or a flow cytometry based selection scheme to screen specific antibody-like scaffolds against specific targets. Again, it should be emphasized that it will be possible to generate protein ligands against any surface epitope on any pathogen protein.

A structure-based approach will also be developed for selecting the CDR loops that specifically target a given surface epitope on a toxin with a known three-dimensional structure, such as anthrax PA or *Botulinum* Neurotoxins A/B. For this, we will compare (by BLAST) the surface loops of a given pathogen protein with the known corresponding antigen sequences in the antibody/antigen database. Next, we will identify the antigen sequence that is most homologous to a given surface epitope of a pathogen protein. Finally, we will incorporate the CDR loops of the corresponding antibody in the database in our “antibody-like scaffold”. Further refinement of the CDR loop sequences will be achieved by molecular dynamics simulated annealing (MDSA) through optimization of the energy of interaction between the ligand and the surface epitope of the pathogen protein [9, 10].

For the reasons described above, “antibody-like scaffolds” and bispecific antibodies can both be designed to have high affinity and specificity against their target protein. Affinities and specificities can

further be improved by constructing bispecific "antibody-like scaffolds" against a target protein. These protein ligands with high affinities and specificities against toxins or virulent factors will have dual purpose in that they can used in any biosensor platform for rapid and sensitive detection or they can be used as effective therapeutics with the aid of appropriate delivery vehicles.

Acknowledgment
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1. Finkelstein, A., *The channel formed in planar lipid bilayers by the protective antigen component of anthrax toxin.* Toxicology, 1994. **87**(1-3): p. 29-41.
2. Pezard, C., P. Berche, and M. Mock, *Contribution of individual toxin components to virulence of Bacillus anthracis.* Infect Immun, 1991. **59**(10): p. 3472-7.
3. Duesbery, N.S., et al., *Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor [see comments].* Science, 1998. **280**(5364): p. 734-7.
4. Leppla, S.H., *Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells.* Proc Natl Acad Sci U S A, 1982. **79**(10): p. 3162-6.
5. Vitale, G., et al., *Anthrax lethal factor cleaves the N-terminus of MAPKKS and induces tyrosine/threonine phosphorylation of MAPKS in cultured macrophages [In Process Citation].* J Appl Microbiol, 1999. **87**(2): p. 288.
6. Cirino, N.M., et al., *Disruption of anthrax toxin binding with the use of human antibodies and competitive inhibitors.* Infect Immun, 1999. **67**(6): p. 2957-63.
7. Shyjan, A.M., et al., *Human mucosal addressin cell adhesion molecule-1 (MAdCAM-1) demonstrates structural and functional similarities to the alpha 4 beta 7-integrin binding domains of murine MAdCAM-1, but extreme divergence of mucin-like sequences.* J Immunol, 1996. **156**(8): p. 2851-7.
8. Briskin, M., et al., *Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue.* Am J Pathol, 1997. **151**(1): p. 97-110.
9. Catasti, P., et al., *Structure-function correlations of the insulin-linked polymorphic region.* J Mol Biol, 1996. **264**(3): p. 534-45.
10. Catasti, P., E.M. Bradbury, and G. Gupta, *Structure and polymorphism of HIV-1 third variable loops.* J Biol Chem, 1996. **271**(14): p. 8236-42.